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Microsatellite and mitochondrial DNA homogeneity among phenotypically diverse crossbill taxa in the UK

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Genetic differentiation within and between the three morphologically divergent crossbill species extant in the UK was assessed by comparison of allele frequencies at five unlinked microsatellite loci and DNA sequence variation across the mitochondrial control region. No significant differences in microsatellite allele frequency were found either between different populations of the same species or between the crossbill species themselves. A similar lack of genetic divergence was apparent from the mitochondrial sequence data. We resolved 33 different haplotypes, separated by low levels of sequence divergence (0–0.15%). Levels of divergence within and between species were not significantly different. Haplotypes formed a polyphyletic phylogeny, indicating that the crossbill species do not form genetically separate clades. Discordance between neutral DNA polymorphisms and adaptive morphological variation is discussed in relation to defining the systematic relationship between crossbill forms. If adaptive differences have arisen without a concomitant divergence in neutral DNA then attempting to define crossbill types from microsatellite and mitochondrial DNA without recourse to ecology and behaviour may be misleading.

Keywords: crossbill; DNA; hybridization; *Loxia*; speciation

1. INTRODUCTION

An increasing number of studies, from a diverse range of taxa, are showing that natural selection caused by shifts in ecology can cause extremely rapid adaptive morphological divergence, frequently in the absence of discernable differentiation at neutral DNA loci (Orr & Smith 1998). Such discrepancies between adaptive and neutral characters are providing considerable insight into the relative contributions of stochastic and deterministic evolutionary processes in the early stages of speciation. They also highlight some potential problems with the pervasive trend toward using DNA data to define species and resolve systematic relationships (e.g. Cracraft 1983), and to help to prioritize conservation effort (Crandall *et al.* 2000). Ultimately, rapidly evolving polytypic species can be acting as true species, either in allopatry or sympatry, but will not be recognized as such by species definitions that require discernible genetic identities, which are invariably assessed by screening neutral DNA polymorphisms.

Here, we highlight how discordance between morphological and genetic character sets can confuse taxonomic issues, by describing the genetic relationships among crossbill taxa in the UK.

Three species of crossbill breed in the UK: the common crossbill (*Loxia curvirostra*), which has a widespread distribution; the parrot crossbill (*Loxia pytyopsittacus*), which is an occasional breeder; and the Scottish crossbill (*Loxia scotica*). These species are defined primarily according to bill depth, with differences being a product of adaptation to specialist feeding on particular types of conifer cone (Lack 1944; Benkman 1993). *L. curvirostra* has a slender bill to extract seeds from the small cones of the Norway spruce (*Picea abies*), whereas parrot crossbills have much deeper bills to extract seeds from the larger more robust cones of the Scots pine (*Pinus sylvestris*). Birds resident in north east Scotland that are considered to inhabit old Scots pine plantations and the pockets of semi-natural pinewood that represent the remnants of the Caledonian forest have bill depths intermediate between those of common and parrot crossbills, and have been designated as Scottish crossbills. Whilst the distributions of bill sizes for common and parrot crossbills are distinct, the bill distribution for Scottish crossbills overlaps both the common-crossbill and the parrot-crossbill distributions. This makes it difficult to identify crossbills in the field and provide accurate population size estimates for the three forms, and has led to considerable debate over the taxonomic status of the Scottish crossbill. Different authorities have classified the Scottish crossbill as a subspecies of the common crossbill, a subspecies of the parrot crossbill, a temporal pseudospecies and, latterly, a full species (see Knox 1975). This full-species status makes the Scottish crossbill the UK's only endemic bird species (BOURC 1980 (British Ornithological Union Records Centre)). There is no documented evidence of mixed breeding pairs, which may indicate that behavioural or ecological isolation is maintaining species integrity between the crossbill forms (Knox 1990). However, as a result of the recent planting of non-native spruces, combined with irruptive invasions of crossbills from Continental Europe following the failure of cone crops, there is an increasing occurrence of sympatric breeding of the three crossbill types in Scotland (Jardine 1992; Summers *et al.* 2001), which may enhance the opportunity for introgressive hybridization.

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Hitherto, there has been no description of the genetic relationships between crossbills in the UK. Theory predicts that if crossbill forms have been reproductively isolated for a considerable period then each should form a distinct genetic grouping. In this study, we determined genetic divergence within and between crossbill types from sequence variation at the mitochondrial control region and allelic variation at several hypervariable microsatellite loci. Specifically, we tested whether, first, microsatellite loci show significant homogeneity in allele frequencies across crossbill forms, and second, crossbill forms are reciprocally monophyletic in mitochondrial DNA phylogenies.

2. MATERIAL AND METHODS

(a) *Sampling*

A blood spot and/or feather sample, together with a measurement of minimum bill depth, were taken from 503 adult crossbills caught by live trapping from various populations in the UK plus an outgroup population from Continental Europe. All sampling was non-destructive and performed under licence where required.

Birds were assigned to species by bill depth. However, given the overlap in bill-depth distributions between *Loxia* species, only those individuals whose bill depths fell below the published modal value for common crossbills (the small-billed species), close to the accepted modal value for Scottish crossbills, or above the modal value for parrot crossbills (the large-billed species) were used in subsequent analyses. The published mean bill depths for common, Scottish and parrot crossbills are 10.5 mm, 11.5 mm and 13.4 mm, respectively (Knox 1976). The individuals chosen here had bill depths of less than 10.2 mm, 11.3–11.7 mm or more than 13.4 mm.

(b) *Microsatellite genotyping*

A total of 274 crossbills were scored at five microsatellite loci. Primer sequences, DNA extraction procedures and polymerase chain reaction (PCR) protocols are provided in Piertney *et al.* (1998). The total sample comprised 163 common, 46 parrot and 65 Scottish crossbills. Each 'species' sample could be further subdivided into several 'population' samples according to the following localities: common crossbills from Ballater (57°3′ N, 3°3′ W, $n = 34$), Pyrenees ($n = 23$), Scottish borders ($n = 27$), Glen Tanar (57°3′ N, 2°52′ W, $n = 22$), Inver (57°2′ N, 3°18′ W, $n = 35$) and Kielder forest (55° N, 2° W, $n = 22$); Scottish crossbills from Ballater ($n = 14$), Glen Tanar ($n = 11$), Abernethy (57°15′ N, 3°40′ W, $n = 21$) and Inver ($n = 19$); and parrot crossbills from Mar Lodge (57°2′ N, 3°35′ W, $n = 14$), GlenTanar ($n = 17$) and Abernethy ($n = 15$).

To quantify genetic divergence between samples we performed an analysis of molecular variance (AMOVA) (Excoffier *et al.* 1992) using ARLEQUIN 1.1 software (Sneider *et al.* 1997). Such an analysis permits a hierarchical examination of structure (measured as Weir & Cockerham's (1984) analogue of F_{ST}), partitioning variance in this case between crossbill species irrespective of geographic location, between populations of the same crossbill species and between all populations, irrespective of species.

(c) *Mitochondrial sequence analysis*

In total, 50 adult individuals were screened for DNA sequence variation across the complete mitochondrial control region. These comprised common, Scottish and parrot crossbills ($n = 18$, $n = 11$ and $n = 17$, respectively) from UK populations, common crossbills from the Pyrenees ($n = 3$) and a single outgroup sample of a two-barred crossbill (*Loxia leucoptera*) from Russia.

The control region was amplified in two segments using two pairs of the universal passerine PCR primer sets described in Tarr (1995) and reaction conditions given in Piertney *et al.* (1998). The first pair were L16743 (5′-TTCTCCGAGATC-TACGGCCT-3′) and H417 (5′-ATGAGCTCGGTTCTCGT GAG-3′) and the second pair were L437 (5′-CTCACGAGAA CCGAGCTACT-3′) and H1248 (5′-CATCTTCAGTGTATGCT-3′). These two reactions gave PCR products of approximately 500 base pairs (bp) and 800 bp, respectively.

PCR products were purified using Qiagen purification columns (Qiagen, UK), then sequenced using an ABI PRISM 377 automated DNA sequencer (PE-Applied Biosystems UK) (dye-terminator cycle sequencing according to the manufacturer's instructions).

Sequences were aligned using CLUSTAL (Higgins *et al.* 1992), and evolutionary distance between haplotypes (measured as the number of nucleotide substitutions per site) was calculated using the Tamura–Nei model (Tamura & Nei 1993) within MEGA (Kumar *et al.* 1993). Gamma rate variation between sites was set at $\alpha = 0.11$, according to Kocher & Wilson (1991) and Tamura & Nei (1993).

A phylogeny of unique haplotypes was constructed from the calculated Tamura–Nei distances using the neighbour-joining approach. Bootstrap resampling, with 1000 replicates, was used to examine the stability of internal groupings.

Genetic differentiation between the three putative species was assessed by calculating K_{ST} (Hudson *et al.* 1992), the significance of which was determined by permutation using the Proseq package (D. Filatov, unpublished data).

3. RESULTS

(a) *Microsatellite DNA variation*

All five microsatellite loci examined displayed high levels of genetic variation. The mean numbers of alleles per locus (N_a) for the common, Scottish and parrot samples were 21, 13 and 14, respectively, and mean observed heterozygosities (H_o) were 0.76, 0.82 and 0.73, respectively. When the effects of unequal sample size were removed, there were no significant differences in N_a or H_o between crossbill species (Monte Carlo permutation test, $p < 0.05$). The majority of alleles were shared both between populations and between species. The few alleles resolved in only one crossbill species were observed only at extremely low frequencies and were not present in every population of that particular species. No single- or multi-locus deviations from Hardy–Weinberg expectations were observed within any population or putative species type (exact probability test, $p > 0.05$).

The AMOVA indicated no genetic structure among populations within a particular species, and no divergence between crossbill species (table 1). Out of the total genetic diversity, only 0.49% was attributable to differences between crossbill species and 0.19% to differences between populations within species. The vast majority (99.31%) was attributable to differences between individuals within a population.

(b) *Mitochondrial DNA sequence variation*

A total of 1140 nucleotides could be unambiguously resolved from all 50 individuals examined. A total of 42

Table 1. Analysis of molecular variance (AMOVA) for 11 crossbill populations categorized into three putative species based on weighted average *F* over five polymorphic microsatellite loci (Weir & Cockerham 1984)

source of variation	d.f.	sum of squares	variance components	percentage of variation
among species	2	1.4	0.00216	0.49
among populations within species	9	4.2	0.00086	0.19
within populations	434	190.1	0.43805	99.31
total	445	195.7	0.44108	

Table 2. Polymorphic nucleotide sites defining the 33 control-region haplotypes resolved from the 50 crossbills examined

(Sequences are given in relation to haplotype 1, where a dot indicates identity. The putative crossbill type that the haplotype was found in is provided. C: common crossbill; S: Scottish crossbill; P: parrot crossbill; TB: two-barred crossbill. The haplotype 1 sequence is deposited in the Genbank nucleotide database under accession number AY029371.)

	1	26	44	150	168	173	195	209	248	272	293	308	342	344	429	433	494	499	545	588	611	663	705	811	817	837	845	851	895	912	916	922	959	976	1018	1031	1062	1066	1074	1114	1134	1136	
1	T	C	C	T	C	T	G	G	T	A	T	C	A	A	G	C	C	T	C	C	T	A	T	A	G	T	C	G	T	G	A	C	C	A	C	G	T	A	C	C	A	A	C
2	.	.	.	C	.	.	A	.	.	.	.	T	.	.	A	.	.	.	.	.	C	T	.	.	.	.	.	.	.	C	.	.	.	.	.	A	.	G	T	T	.	.	C
3	.	.	.	C	.	.	A	.	.	.	.	T	.	.	A	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S
4	.	T	.	.	.	.	.	.	.	.	.	.	.	G	A	.	.	.	G	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	S
5	.	T	.	.	.	.	.	.	.	.	.	.	.	G	A	T	.	.	G	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	G	T	.	.	.	S
6	.	T	.	.	.	.	.	.	.	.	.	.	.	G	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C,C,S,C
7	.	T	.	.	.	.	A	.	.	.	.	T	.	.	.	.	.	.	.	.	C	T	.	.	.	.	.	A	.	C	.	.	.	.	.	.	.	G	T	.	.	.	P
8	.	T	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	C	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	P
9	.	T	.	.	.	.	.	.	.	.	.	T	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	P,P
10	.	T	.	.	.	.	.	.	.	.	.	T	.	.	A	.	.	.	.	.	C	T	.	.	.	.	.	.	.	C	.	.	.	.	.	A	.	G	T	.	G	.	S
11	.	T	.	.	.	.	A	.	.	.	.	T	.	.	A	.	.	.	.	.	C	T	.	.	.	.	.	.	.	C	.	.	.	.	.	A	.	G	T	.	.	.	C
12	.	T	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	P,P
13	.	T	.	.	.	.	A	.	.	.	.	.	.	.	A	.	.	.	G	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	P
14	.	T	.	.	.	C	A	.	.	.	.	T	.	.	A	.	.	.	.	.	.	T	.	.	.	.	.	.	.	C	.	.	.	.	.	A	.	G	T	.	.	.	P
15	.	T	.	.	.	.	A	.	.	.	.	T	.	.	A	.	.	.	.	.	C	T	.	.	.	.	.	.	.	C	.	.	.	.	.	A	.	G	T	.	G	.	S,C
16	.	T	.	.	.	.	A	.	.	.	.	.	.	G	A	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	S
17	.	T	T	.	.	.	.	.	.	.	.	.	.	G	A	.	.	C	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S
18	.	T	.	.	.	.	.	.	.	.	.	.	.	G	A	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S,P,S,S,C
19	.	T	.	.	.	.	.	.	.	.	.	T	.	G	A	.	.	.	G	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	T	T	.	.	S
20	.	T	.	.	.	.	.	.	.	.	.	.	.	G	A	.	.	.	G	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	G	T	.	.	.	S
21	.	T	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S
22	.	T	.	.	.	.	A	.	C	.	.	T	.	.	A	.	.	.	.	.	C	T	.	.	.	.	.	.	.	C	.	.	.	.	.	A	C	G	T	.	.	.	C,S,S
23	.	T	.	.	.	.	A	.	.	G	.	.	.	G	A	.	.	.	G	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	C
24	.	T	.	.	.	.	A	.	.	.	.	T	.	.	A	.	.	.	.	.	C	T	.	.	.	.	.	A	.	C	.	.	.	.	.	.	.	G	T	.	.	.	C,C,C
25	.	T	.	.	.	.	.	.	.	.	.	.	.	G	A	.	T	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C
26	.	T	.	.	.	.	A	A	.	.	.	T	.	.	A	.	.	.	.	.	C	T	.	.	.	.	.	.	.	C	.	.	.	.	.	A	.	G	T	.	G	.	C
27	.	T	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	C	T	.	.	.	.	.	.	.	C	.	.	.	.	.	A	.	G	T	.	.	.	C
28	C	T	T	.	.	.	A	.	.	.	.	T	.	.	.	.	.	.	.	.	C	T	.	.	.	.	.	.	.	C	.	.	.	.	.	A	.	G	T	.	.	.	C
29	.	T	T	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	G	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	C
30	.	T	.	C	T	.	.	.	C	.	C	T	G	.	A	.	T	.	.	T	.	.	A	G	A	C	T	.	C	.	G	T	T	G	T	.	.	.	.	T	.	G	TB
31	.	T	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	P,C,S
32	.	T	.	.	.	.	A	.	.	.	.	.	.	G	A	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C,P
33	C	T	.	.	.	.	.	.	.	.	.	.	.	G	A	.	.	.	.	.	C	T	.	.	.	.	.	.	.	C	.	.	.	.	.	A	.	G	T	.	.	.	C

variable sites were observed, which defined 33 different haplotypes (table 2). Out of these 42 variable sites, 38 involved transition mutations, three involved transversion mutations and one site showed both a transition and a transversion.

Some of the haplotypes were shared by more than one individual and in several cases between species (e.g. haplotype 18 was found in three Scottish crossbills, one common crossbill and one parrot crossbill).

The evolutionary relationships between the haplotypes are shown in figure 1. The salient feature of this topology is that the species do not form monophyletic clades. Polyphyly among the crossbill types was also apparent when other phylogenetic algorithms were used (i.e. maximum parsimony and maximum likelihood) and when different models of sequence evolution (F84, K2P and HKY85) were assumed (phylogenies not shown). The likelihood of the resolved topology was significantly

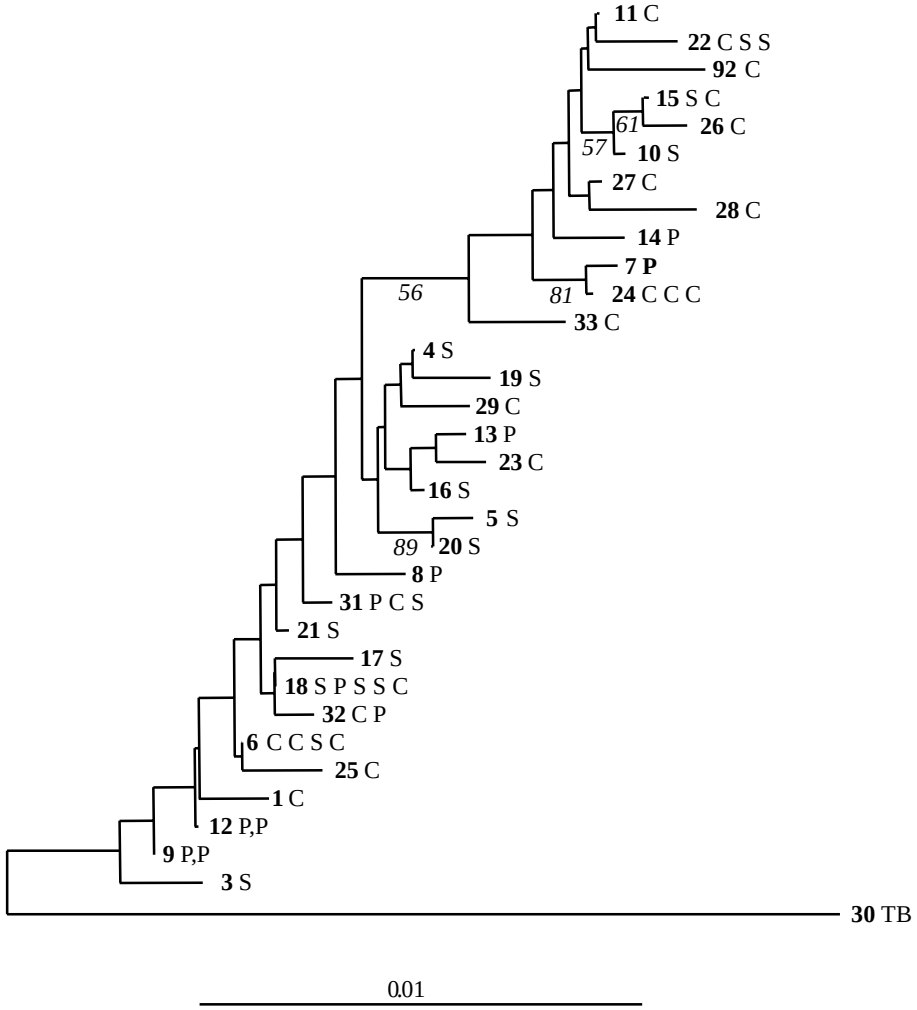

Figure 1. Neighbour-joining phylogeny of the 33 crossbill mitochondrial sequences based on the Tamura–Nei distance with a gamma shape parameter of 0.11. The letters at the tips of the branches indicate in which putative crossbill species that particular haplotype was resolved (C: common crossbill; S: Scottish crossbill; P: parrot crossbill; TB: two-barred crossbill). Bootstrap confidence indexes $>50\%$ are provided at each internal branch.

greater than a topology constrained to be monophyletic for the three putative forms (Shimodaira–Hasegawa test, $p < 0.05$).

Levels of sequence divergence among crossbills of the same species ranged from 0 to 0.0137 (mean = 0.00631, 95% confidence interval = 0.0062–0.0067), and between crossbill types from 0 to 0.0148 (mean = 0.00640, 95% confidence interval = 0.0062–0.0067). The frequency distributions of sequence divergence within and between crossbill species are shown in figure 2.

No significant population differentiation was observed between the three putative species ($K_{ST} = 0.0005$, $p = 0.51$).

4. DISCUSSION

This study has shown that the crossbill species extant in the UK, which show considerable variability in bill shape, are genetically indistinguishable at several independent microsatellite and mitochondrial loci.

The high levels of allelic variation inherent in microsatellite markers make them particularly useful for detecting genetic divergence between recently derived or geographically proximate populations, when differences may be limited. The minimal level of divergence detected in this study, both between populations of the same putative crossbill type and between crossbill types, indicates genetic homogeneity associated with prevalent gene flow. High levels of gene flow are easy to reconcile within crossbill species, given the nomadic nature of populations (Newton 1972; Questiau *et al.* 1999). However, an equivalent lack of structure between crossbill species implies interbreeding. The propensity for irruptive invasion of common and parrot crossbills from continental Europe following the failure of conifer cone crops (Newton 1972) would provide the opportunity for sympatric breeding. To date, however, no documented cases of interbreeding exist and ongoing studies are finding no heterospecific pairs in natural populations (R. Summers, unpublished data). A lack of observed hybridization may partly reflect the difficulties associated with identifying heterospecific pairs in the field.

The lack of divergence at microsatellite loci is also reflected in the mitochondrial data. Levels of sequence divergence between haplotypes resolved from different crossbill types are comparable with those within crossbill types, and several haplotypes were shared between individuals of different putative species. Mitochondrial

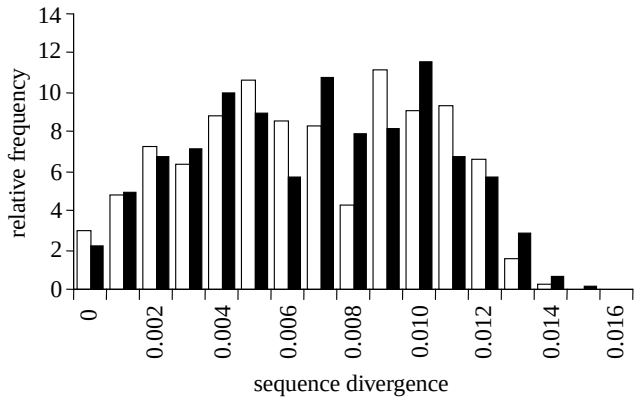

Figure 2. Frequency distributions of nucleotide divergences between individuals of the same (white bars) and different (black bars) crossbill type.

haplotypes can be shared between crossbill forms because of either incomplete lineage sorting or introgressive gene flow. After divergence from a single ancestral form, species are expected initially to share a number of mitochondrial haplotypes, but over time there will be stochastic loss of certain haplotypes from some populations until each has a unique composition of mitochondrial types. In a phylogenetic context, there will be a gradual progression from polyphyly through paraphyly to monophyly. If Scottish, common and parrot crossbills are reproductively isolated, but diverged relatively recently, then the current survey will have characterized the patterns of mitochondrial variation during the earlier stages of polyphyly or paraphyly.

Alternatively, the extant pattern of haplotype sharing could be due to introgressive gene flow between crossbill forms. Avise (1989) reviewed patterns of gene flow inferred from phylogenetic and phylogeographical structuring of mitochondrial sequences. By reviewing numerous studies, four patterns were defined: first, high mitochondrial sequence divergence ($> 2.0\%$) coupled with geographical structure; second, high mitochondrial divergence but no geographical structure; third, low divergence ($< 1\%$) with geographical structure; and fourth, low divergence with no geographical structure. The extremely low levels of mitochondrial divergence found in this study, together with a mixed haplotype distribution across populations, indicate that crossbill forms fall into the fourth category. In Avise's review this category is occupied solely by species with life histories conducive to dispersal with ranges free from impediments to gene flow (e.g. insects, birds and marine fish).

Separating the contributions of introgressive gene flow and incomplete lineage sorting in maintaining polyphyletic phylogeny is problematic. In this case we cannot exclude the possibility of incomplete lineage sorting, especially given the relatively short branch lengths resolved in the phylogeny. Neigel & Avise (1986) have estimated that the number of generations required for a population to reach monophyly is roughly four times the effective population size, though processes such as population bottlenecks will dramatically reduce the time to monophyly. If it is assumed that the Scottish crossbill diverged from the ancestral crossbill in isolation in the Caledonian forest, as postulated by Knox (1976), then the onset of divergence could have occurred 10 000–27 000 years ago, during the last glaciation, in a refugium thought to have existed in the north of Ireland (Bennett 1984). This would mean that the population would have to have been maintained at levels in excess of 2500–7000 breeding females to prevent monophyly being reached. Providing accurate historical population sizes for the Scottish crossbill is extremely difficult but the maximum population size can be interpolated from current densities and the known historical extent of the Caledonian forest. Nethersole-Thompson (1975, p. 177) reported inter-nest distances of between 50 yards and 400 yards in years of peak density and up to 1 mile in years of low density, which equates to approximately one breeding pair per 2 km^2. The maximum extent of the Caledonian forest was approximately 15 000 km^2 some 5000 years before present (Bain & Bainbridge 1988). Such a forest could, thus, have sustained approximately 7500 pairs of crossbills, which might be sufficient to maintain polyphyly between crossbill forms. However, there is evidence that the Scottish crossbill population has undergone severe contraction, so this population estimate is a maximum and could not have been maintained after the end of glaciation. From Neolithic times, and particularly during the Dark Ages, the Caledonian forest was cleared for agriculture, and this, combined with climatic deterioration, reduced the habitat to about 160 km^2 (Forest Authority 1994; Tipping 1994). This reduction would have produced a considerable population bottleneck, and indeed Nethersole-Thompson (1975) produced a population-size estimate of 1500 adult individuals in the most productive years in the 1970s. A population bottleneck of this magnitude should have resulted in the formation of a monophyletic Scottish crossbill clade with respect to common and parrot crossbills.

A lack of genetic distinction between the crossbill forms could argue against the Scottish crossbill warranting full species status. The biological species concept dictates that species should form groups of interbreeding individuals that are each reproductively isolated from other such groups. If introgressive gene flow occurs then this criterion is not met. Irrespective of levels of gene flow, a polyphyletic phylogeny also precludes full species status under the phylogenetic species concept (Cracraft 1983), which requires that species have discernible genetic signatures (reciprocal monophyly). Concepts such as 'evolutionarily significant unit', which were developed to provide a rational basis for prioritizing taxa for conservation effort divorced from traditional taxonomic categorization, would also fail to recognize the Scottish crossbill as having an independent evolutionary heritage. Evolutionarily significant units are defined as being 'reciprocally monophyletic for mtDNA alleles, and show significant divergence in allele frequencies at nuclear loci' (Moritz 1994).

However, if all these definitions fail to recognize the Scottish crossbill as a separate species, they also fail to recognize common and parrot crossbills as separate species, as they themselves are not reciprocally monophyletic. Yet the specific status of these two forms is not in debate and no evidence exists of interbreeding between parrot and common crossbills in sympatric populations from Europe.

The lack of distinct genetic signatures for common and parrot crossbills highlights the generally low levels of genetic divergence between crossbill morphotypes, which therefore should not be used as a gauge for defining management and species units in other congeners.

Indeed, the level of variation observed in this study, together with its partitioning between morphologically divergent groups, is increasingly being observed across a range of taxa (e.g. Orr & Smith (1998) and references therein) and this phenomenon appears particularly common in passerine birds (see Seutin *et al.* (1995) and references therein; Questiau *et al.* 1999; Freeland & Boag 1999). In some cases this has been attributed to occasional hybridization (Freeland & Boag 1999) but increasingly the effects of selection in generating rapid divergence irrespective of levels of gene flow have been highlighted (Smith *et al.* 1997).

If selection does affect adaptive divergence sufficiently for morphological variation to be maintained in the face of gene flow then assessment of phylogenetic relationships from neutral DNA polymorphisms can generate spurious conclusions. The original definitions of management units and species units had two components: reproductive and historical separation, and adaptive distinctiveness. With the pervasive application of molecular genetic techniques, efforts to document evolutionarily significant units have emphasized reproductive isolation rather than the maintenance of adaptive differences. This can be difficult to reconcile given that, for a particular species, evolutionary success is maximized through the maintenance of adaptive diversity.

In the case of the Scottish crossbill, an endemic morphological form exists that shows adaptive divergence in a particular ecological niche (Marquiss & Rae 2001). Such adaptive differences may have arisen without concomitant divergence in neutral DNA variants and, as such, attempting to define the Scottish crossbill from microsatellite and mitochondrial variation alone, without recourse to ecology and behaviour (Summers *et al.* 2001), can be misleading and could undermine the importance of conserving morphological diversity when maintaining local biodiversity.

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